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$\alpha_v\beta_3$ integrin engagement enhances cell invasiveness in human multiple myeloma

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Background and Objectives. In multiple myeloma (MM), the mechanisms used by plasma cells to invade locally and metastasize are thought to be similar to those developed by solid tumors and include cell proliferation and secretion of extracellular matrix (ECM)–degrading enzymes following adhesion to ECM proteins. We studied these mechanisms in fresh bone marrow plasma cells of patients with MM after adhesion to the ECM proteins vitronectin (VN) and fibronectin (FN).

Design and Methods. The ability of bone marrow plasma cells to adhere to VN and FN and the consequent formation of focal adhesion plaques on the cell surface, their composition and phosphorylation of several signal transduction proteins, cell proliferation and secretion of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) and urokinase-type plasminogen activator (uPA) were studied.

Results. Plasma cells adhered to immobilized VN and FN. Adhesion was fully prevented by neutralizing anti-$\alpha_v\beta_3$ integrin antibody. Integrin engagement caused aggregation of the plaques, which contained the β_3 integrin subunit, some cytoskeletal proteins, tyrosine kinases, the Grb-2 adapter protein, and mitogen-activated protein (MAP) kinase. Free and immobilized VN and FN stimulated cell proliferation and the production and the release of uPA, and increased the release of the activated forms of MMP-2 and MMP-9 in an $\alpha_v\beta_3$ integrin-dependent manner.

Interpretation and Conclusions. This ability of myeloma plasma cells to interact with VN and FN via $\alpha_v\beta_3$ integrin engagement suggests a novel mechanism for their invasion and spreading, since this interaction allows them to adhere to the substratum and enhances their proliferation and protease secretion.

Key words: extracellular matrix, focal adhesion, matrix metalloproteinases, multiple myeloma, urokinase-type plasminogen activator.

Cell interaction with the extracellular matrix (ECM) is a mandatory event for various biological processes, including cell proliferation, differentiation, migration and death, and for tumor growth, invasion and metastasis.[1,2] Integrins are the major family of cell surface receptors that mediate cell adhesion to several ECM proteins, including vitronectin (VN) and fibronectin (FN).[3] Integrin engagement leads to the formation of focal adhesion contacts or plaques, generated by a cascade of phosphorylation events that give rise to the recruitment and assembly of actin-binding proteins, with the consequent linking of the ECM proteins on the extracellular side of the cell membrane to cytoskeletal proteins and actin filaments on the cytoplasmic side.[4,5] This causes the activation (or phosphorylation) of intracellular signal transduction pathways, leading to integrin-mediated changes in cell behavior and gene expression.[5-7]

In endothelial cells, fibroblasts, macrophages, platelets, and solid tumor cells, integrin engagement triggers the patchy transmembrane accumulation and phosphorylation of many signal transduction molecules, including tyrosine kinases (e.g. FAK and pp60^{src}), Grb-2 adapter protein, the Sos activator of the Ras pathway, and mitogen-activated protein (MAP) kinase ERK-2. The parallel tyrosine phosphorylation of some cytoskeletal proteins, such as vinculin and paxillin, cooperates in the localized construction of the signaling complexes in focal adhesion plaques.[8-13] Many signaling proteins activated by integrin engagement are also involved in signal transduction pathways activated by growth factor receptors.[14] Thus, it is not surprising that cell-ECM interaction profoundly affects the response of target cells to mitogenic stimuli.

Integrin-dependent interaction with ECM proteins plays an important role in modulating the pro-

liferation of tumor cells and their ability to release ECM-degrading enzymes, including matrix metalloproteinase (MMP)-2 and MMP-9, and urokinase-type plasminogen activator (uPA), as shown for melanoma,[15,16] and breast,[17] small-bowel,[18] colon[19] and ovary[20] carcinomas. This has suggested that ECM interaction is crucial for the growth of solid tumors and their ability to invade and metastasize.

We have shown that plasma cells from patients with multiple myeloma (MM) constitutively secrete MMP-2 and MMP-9 and have angiogenic ability.[21] We have also shown that MM and acute leukemia B-cell and T-cell lines adhere to VN and FN via $\alpha_v\beta_3$ integrin, which increases their proliferation and MMP-uPA secretion.[22] Little is known about these relations in fresh bone marrow plasma cells from MM patients. There is evidence that the interaction of plasma cells with the ECM mediates their multidrug resistance[23,24] and homing to the bone marrow.[25] Here we show that plasma cells left to interact with VN and FN engage $\alpha_v\beta$ integrin and form plaques in which several signal transduction proteins and the MAP kinase ERK-2 in its phosphorylated form are recruited, and enhance proliferation and MMP-uPA secretion.

Design and Methods

Cells

Bone marrow plasma cells from 14 patients with newly-diagnosed MM were obtained as described previously.[21] Briefly, bone marrow aspirates were subjected to Ficoll-Hypaque density gradient centrifugation and plasma cell enrichment by removal of T-cells (with two-fold E-rosetting) and monocytes/macrophages (with plastic adhesion). Enriched plasma cells were then obtained by incubating residual cells with magnetic beads (Oxoid Dynal, Oslo, Norway) coated with an antibody to the plasma cell marker CD38 (Becton Dickinson, Mountain View, CA, USA), magnetic subtraction and bead detachment. Enriched plasma cells contained less than 2% of T-cells and monocytes, as assessed by flow cytometry and with the anti-CD3 and anti-CD68 antibodies, respectively (FACScan, Becton Dickinson). They consisted of more than 95% tumor plasma cells and their clonally related cells, as assessed by morphology in May-Grünwald-Giemsa stains and flow cytometry with the anti-CD38 antibody, or by immunocytochemical staining with anti-κ or anti-λ antibody (Dako, Glostrup, Denmark) according to the light chain of the M-component.[21] Cells were cultured in complete medium: RPMI-1640 medium containing 1% glutamine, 10% heat inactivated fetal calf serum (FCS), 100 U/mL penicillin and 50 mg/mL streptomycin (all from Gibco, Life Technologies Ltd., Paisley, UK); their viability assessed by trypan blue exclusion was greater than 90%.

Antibodies

Murine monoclonal anti-$\alpha_5\beta_1$ and anti-α_5 (both from Chemicon International Inc., Temecula, CA, USA), anti-β_3 and anti-vinculin (both from Sigma Chemical Co., St. Louis, MO, USA), anti-paxillin and anti-FAK (both from Transdaction Labs., Lexington, UK) antibodies, polyclonal (rabbit) anti-pp60^{src}, anti-Grb-2, anti-ERK-2 and anti-phosphorylated ERK-2 (ERK-2-p) antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-mouse or anti-rabbit horseradish peroxidase-labeled antisera (Dako) were used for the Western blots. The LM609 monoclonal anti-$\alpha_v\beta_3$ antibody (Chemicon) was used for inhibition assays. Rabbit anti-β_3 (Chemicon), the mouse anti-paxillin and anti-FAK, and fluorescein isothiocyanate (FITC)-labeled sheep anti-rabbit IgG or FITC- or Texas red-labeled goat anti-mouse IgG (all from Calbiochem, La Jolla, CA, USA) were used for immunofluorescence studies.

Cell-adhesion assay on immobilized VN and FN

Aliquots (100 μL) of 100 mM $NaHCO_3$, pH 9.6 (carbonate buffer), containing 20 mg/μL VN, FN, polylysine (PLL, Sigma) or bovine serum albumin (BSA, Sigma) were added to 96-well polystyrene microtiter plates. Unlike VN and FN, PLL does not engage the $\alpha_v\beta_3$ integrin[8] and was used as the negative control, while BSA was used as an indifferent protein. After 16 h incubation at 4°C, the solution was removed, wells were washed three times with cold phosphate-buffered saline (PBS), overcoated with 3% BSA for 1 h at 37°C, and washed twice with PBS. Then, plasma cells were plated in triplicate (5×10^3 cells/well) in serum-free RPMI 1640 medium (SFM) for 30 min at 37°C in a 5% CO_2 humidified atmosphere, as described elsewhere.[22] In some experiments, cells were seeded in the presence of 5 mg/μL of the anti-$\alpha_v\beta_3$ LM609 antibody. After incubation, wells were washed three times with PBS and the number of adherent cells was estimated by the crystal violet colorimetric assay of Kueng *et al.*,[26] and read at 595 nm in a Microplate Reader 3550 (Bio-Rad Lab., Richmond, CA, USA). The number of adherent cells derived from a calibration curve was expressed as mean ± 1 standard deviation (SD) in two determinations per patient.

Cell proliferation assays

The proliferation of adherent cells was evaluated in short-term cultures by seeding plasma cells

(5×10^3/well) in SFM on the VN-, FN-, or PLL-coated plates (as described above) to allow cell adhesion to the substratum. Adherent cells were counted every 24 h according to Kueng *et al.*[26] The proliferation of cells in suspension was determined by similar seeding of 10×10^3 cells/well on uncoated plates in complete medium (positive control), in SFM (negative control), or in SFM admixed with 1 μg/mL VN, 1 μg/mL FN or both.

The proliferation of cells was inhibited by admixing the medium with 5 μg/mL of the anti-$\alpha_v\beta_3$ antibody. Cells were counted every 24 h in a Bürker chamber and data were expressed as mean ± 1 SD of three determinations per patient.

Western blotting

This was performed as described elsewhere.[27] Briefly, total plasma cell or plaque extracts were subjected to 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing (for the $\alpha_5\beta_1$ integrin heterodimers) conditions.

Gels were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (NEN™, Life Science, Boston, MA, USA), which was incubated for 1 h in 5% non-fat dry milk in tris(hydroxymethyl)aminomethane (TRIS) buffer saline (TBS) with 0.1% Tween 20, washed in the same buffer and incubated with the primary and secondary antibody, each for 1 h at room temperature. After washing in TBS-Tween, the membrane was incubated with enhanced chemiluminescence (NENTM, Life Science), and the signal revealed by exposure to Kodak Biomax film (Eastman Kodak Company, Rochester, NY, USA).

Immunoprecipitation with anti-β_3 antibody

Plasma cells (1×10^6) were incubated with 1 μg/mL VN or FN or with 1 μg/mL PLL (control cells) for 30 min at 37°C. Then, cells were lysed for 20 min in 1 mL of ice-cold lysis buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N'N'-tetracetate [EGTA], 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM sodium vanadate, 2 mM sodium phosphate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL leupeptin, and 100 U/mL aprotinin). Lysates were centrifuged at 12,000 rpm for 15 min and incubated for 2h at 4°C with rabbit preimmune serum (Santa Cruz Biotechnology) and 50 μL of a 50% Protein A-Sepharose slurry (Sigma).

Supernatants were removed and incubated overnight at 4°C with anti-β_3 antibody and Protein A-Sepharose beads. Immunoprecipitates were washed in 20 mM HEPES, pH 7.4, 137 mM NaCl, 1% Triton X-100, 10% glycerol (HNTG buffer), suspended in Laemli's buffer and subjected to 8% SDS-PAGE followed by Western blotting.

Evaluation of MMP-2/MMP-9 and uPA activity release

Plasma cells (1×10^7/T25 flask) were incubated for 24 h at 37°C in 6 mL of SFM in the presence of 1 μg/mL VN, FN or PLL. The supernatant (*conditioned medium* [CM]) was collected under sterile conditions, centrifuged sequentially at 1,200 and 12,000 rpm for 10 min, and filtered through 0.22 μm filters (Costar, Cambridge, MA, USA). Its protein content was evaluated by the Bradford method (Bio-Rad), using BSA as a standard. The released MMP-2 and MMP-9 activities were evaluated by SDS-PAGE gelatin-zymography:[28] 5 μg aliquots of CM proteins were applied to 7.5% SDS-PAGE gels co-polymerized with 0.6 mg/mL type A gelatin from porcine skin (Sigma), electrophoresed in a Protean II dual lab. system (Bio-Rad), washed in 2.5% Triton X-100 for 1 h to remove SDS, incubated for 18 h at 37°C, and stained in 0.1% Coomassie brillant blue.

The gelatinolytic regions were observed as white bands against a blue background. The MMP activity was measured by scoring the intensity of bands as arbitrary optical density (O.D.) units by computerized image analysis (Apple Computer Inc., Cupertino, CA, USA).

For the evaluation of released uPA activity, SDS-PAGE was run under non-reducing conditions as previously described:[22] the aliquots (5 μg) of CM proteins were run on 10% SDS-PAGE, and proteins were then electrophoretically transferred to the PVDF membrane at 400 mA for 2 h in 40 mM sodium phosphate buffer, pH 6.5. Zymography of the transferred proteins was carried out on a casein agarose gel at 37°C for 16 h. The caseinolytic region was seen as a black band against a gray background. Control gel was made in the absence of plasminogen to identify plasminogen-independent caseinolytic activity.

Double immunofluorescence

Plasma cells were allowed to adhere to VN-, FN- or PLL-coated coverslips for 1 h, fixed (4% paraformaldehyde), permeabilized (0.2% Triton X100), blocked (3% BSA in PBS) and then sequentially incubated with the 1:200 diluted rabbit anti-β_3 or mouse anti-paxillin or anti-FAK antibody and 10 μg/mL of the FITC-labeled sheep anti-rabbit IgG of the FITC- or Texas red-labeled goat anti-mouse IgG. Fluorescence was evaluated with a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany).

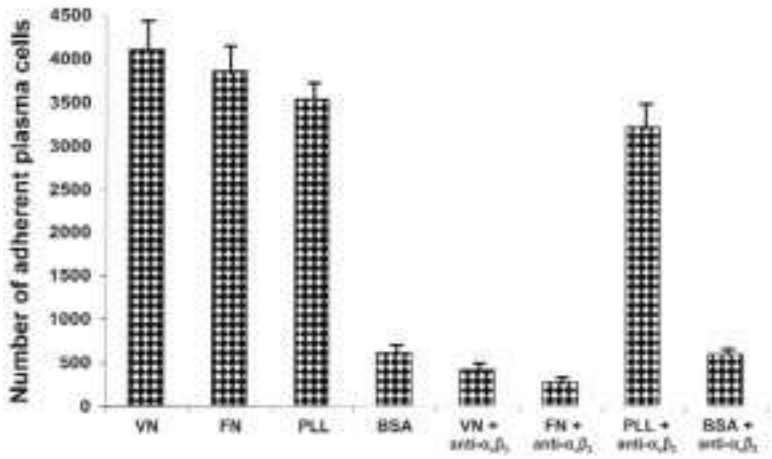

Figure 1. Adhesion of myeloma plasma cells to immobilized VN, FN, PLL and BSA. Plasma cells were incubated under serum-free conditions in plates coated with VN, FN, PLL, or BSA in the absence or presence of neutralizing anti-$\alpha_v\beta_3$ antibody. After 30 min at 37°C, the number of adherent cells per 12.5×10^{-2} mm² was evaluated. Data are the mean ± 1 SD of two determinations per patient in the 14 patients.

Extraction of focal adhesion plaque proteins

This was carried out according to Plopper *et al.*[4] and Del Rosso *et al.*[29] Plasma cells were suspended at 1×10^6/mL in SFM and incubated for 30 min at 37°C with magnetic microbeads (1×10^7/mL, Oxoid Dynal) coated with VN, FN, or PLL in rotation. Microbeads with bound cells were recovered using the side-pull magnetic separation unit (Oxoid Dynal), transferred to 50 μL lysis buffer, and sonicated at 4°C for 10 sec at 10 W. The lysis buffer contains inhibitors of phosphatases, and hence prevents the disgregation of the plaques.[4] Microbeads with bound proteins were then recovered with the magnetic unit, washed three times with HNTG buffer and suspended in Laemli's buffer for electrophoresis. The bound proteins were analyzed by Western blotting as described above.

Results

Bone marrow plasma cells from patients with MM were studied for the formation, protein composition, and functional significance of plaques following cell interaction with VN, FN and control substrate. Similar findings have so far been observed in 14 patients whose plasma cells expressed the $\alpha_v\beta_3$ only. Three patients who expressed $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (VLA-5) integrins simultaneously were excluded to avoid interaction of the latter with FN and VN.

$\alpha_v\beta_3$ integrin mediates plasma cell adhesion to immobilized VN and FN

The ability of plasma cells to adhere to immobilized VN and FN was studied. Cells were plated in SFM in plates coated with VN, FN, PLL (negative control) or BSA (indifferent protein) and the number of adherent cells was quantified after 30 min. More than 80-85% of seeded plasma cells adhered to VN, FN and PLL, whereas adhesion to the BSA was marginal (Figure 1). Western blot analysis of cell extracts showed that the plasma cells expressed the β_3 integrin subunit, but not $\alpha_5\beta_1$ integrin (Figure 2A). This absence was confirmed by Western blot with the anti-α_5 antibody and by flow cytometry with the anti-$\alpha_5\beta_1$ antibody (data not shown). Accordingly, the neutralizing monoclonal anti- $\alpha_v\beta_3$ antibody fully prevented cell adhesion to both VN and FN, but not to PLL (Figure 1). Results indicate that $\alpha_v\beta_3$ integrin mediates the adhesion of MM plasma cells to immobilized VN and FN.

VN and FN induce the formation of plaques by $\alpha_v\beta_3$ integrin engagement

Based on these findings, we wondered whether VN and FN induced the formation of plaques on the cell surface. Integrin-mediated focal adhesion allows grouping into the contact sites of several proteins forming plaques, such as integrins, cytoskeletal proteins, tyrosine kinases such as FAK, MAP kinases and adapter proteins.[4] Figure 3 shows that, after adhesion to VN, plasma cells produced plaques containing the β_3 integrin subunit (panel A), FAK (panels B, E), and the cytoskeletal protein paxillin (panel D), which substantially co-localized (panels C, F), whereas plaques were absent following adhesion to PLL (panels G-I). Overlapping pictures were produced by adhesion to FN (*data not shown*).

The binding of ligand-coated beads is a rapid method for triggering the formation of strong transmembrane adhesive complexes, mediated by cell surface integrin engagement and activation.[4] Plasma cells were therefore incubated with VN- or FN-coated magnetic beads to induce plaque aggregation. They bound to the beads and were then iso-

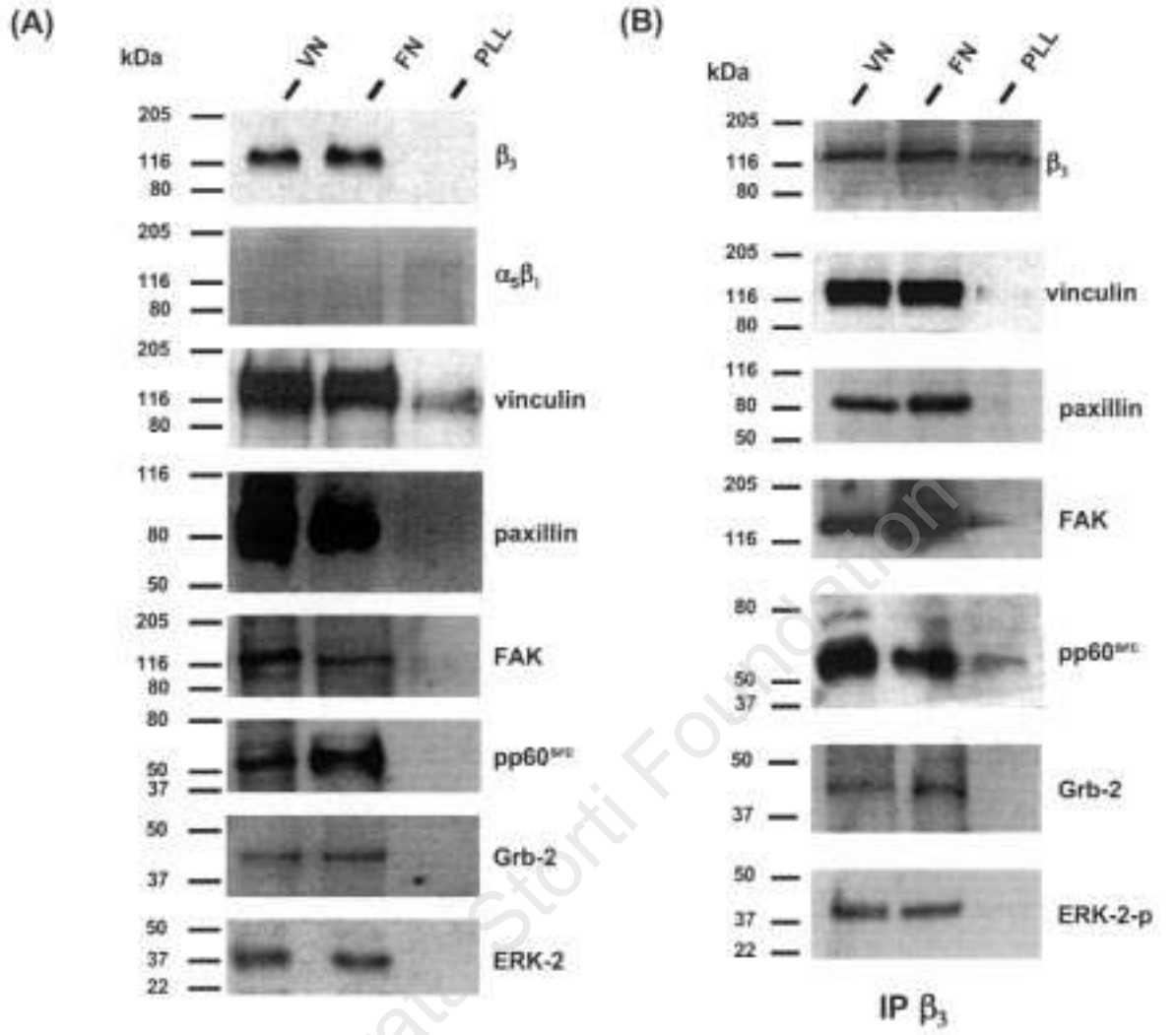

Figure 2. Analysis of focal adhesion plaques induced by VN and FN in myeloma plasma cells. A) Plasma cells from one representative patient were incubated for 30 min at 37°C with magnetic beads coated with VN or FN to induce the aggregation of plaques that bound to the beads and were then isolated and analyzed by Western blotting with the indicated antibodies. PLL-coated beads were used as negative control. B) Plasma cells were allowed to adhere to immobilized VN, FN, or PLL, and after 30 min at 37°C they were lysed, the extracts were immunoprecipitated with anti-β_3 antibody (IPβ_3), and the composition of the immunoprecipitates was analyzed by Western blotting.

lated and analyzed by Western blotting. As shown in Figure 2A, VN and FN rapidly recruited the β_3 integrin subunit, the cytoskeletal proteins vinculin and paxillin, the tyrosine kinases FAK and pp60^{src}, the small adapter protein Grb-2, and the MAP kinase ERK-2 in its phosphorylated form (ERK-2-p). These proteins were not or only marginally found in the material bound to PLL-coated beads. Figure 2B shows that, when extracts were immunoprecipitated with the anti-β_3 antibody and analyzed by Western blotting, they gave co-precipitation with those proteins recruited in the plaques only in cells adherent to VN or FN. These results suggest that plaques are rapidly formed after $\alpha_v\beta_3$ integrin-mediated interaction of MM plasma cells with VN and FN.

VN and FN stimulate plasma cell proliferation

VN and FN, either immobilized[15-18] or in suspension[30, 31] (when they act as polymers),[31] are known to induce proliferation in different cell types. Based on the observation that myeloma plasma cells proliferate spontaneously in short-term culture,[32] we wondered whether VN and FN enhanced prolifer-

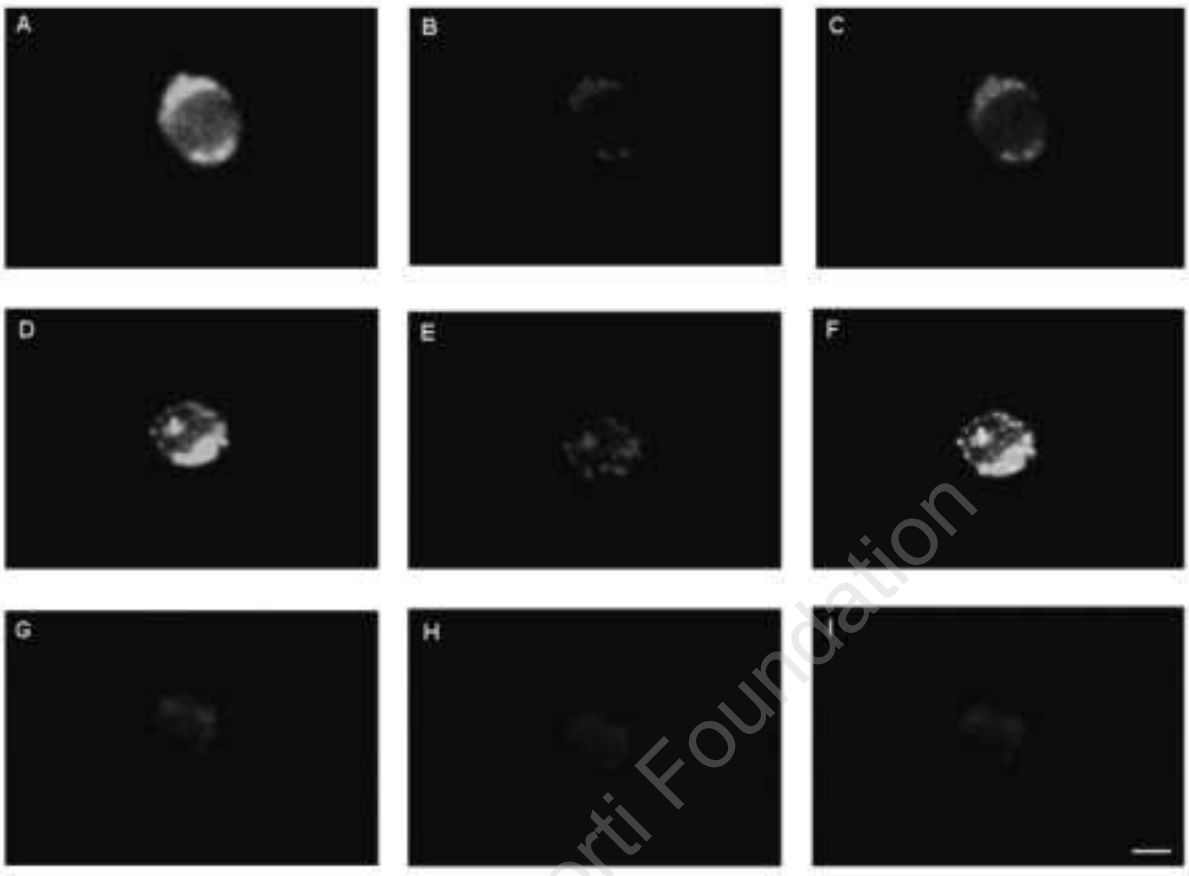

Figure 3. Induction of focal adhesion plaques by VN in myeloma plasma cells. Double immunofluorescence staining in one representative patient shows that adhesion to VN produced plaques containing the β_3 integrin subunit (FITC-staining; A), $p125^{FAK}$ (Texas red-staining; B, E), and paxillin (FITC-staining; D). Double photographs of β_3 subunit and $p125^{FAK}$ (C) and of paxillin and $p125^{FAK}$ (F) showing that the molecules co-localized substantially. No β_3 subunit (G) and $p125^{FAK}$ (H) recruitment (double photograph in I) was observed in cells adherent to PLL. Bar = 7 μm.

ation in freshly-isolated and short-term cultured bone marrow plasma cells from MM patients. Plasma cells in suspension were, therefore, incubated in SFM alone (negative control) or admixed with PLL or with VN, FN, or both, and their growth rate was compared to cell cultures incubated in medium with 10% FCS (positive control). As shown in Table 1, both VN and FN significantly stimulated cell proliferation. When added together, the proteins induced a mitogenic response similar to that exerted by FCS. Proliferation induced by VN, FN or both was strongly inhibited by the anti-$\alpha_v\beta_3$ antibody, thus implying integrin engagement in the mitogenic response. Since the antibody is unable to trigger integrin engagement unless a second (anti-mouse IgG) antibody is subsequently used,[33] we suggest that VN and FN as polymers signal through the $\alpha_v\beta_3$ integrin.

Similar results were obtained when plasma cells were allowed to adhere to immobilized VN or FN in SFM (Table 1): they once again proliferated, whereas those adherent to PLL remained quiescent with signs of distress. Interestingly, the proliferation of adherent cells was similar to that observed for cells grown in suspension under the same experimental conditions. Taken together, these results illustrate the ability of VN and FN to sustain myeloma cell proliferation under serum-free conditions.

VN and FN cause protease release

MM plasma cells constitutively release significant amounts of MMP-2 and limited amounts, if any, of MMP-9, whereas no uPA was detectable in their conditioned medium (CM).[21] Because VN and FN have been shown to cause protease upregulation in solid tumors,[15-20] we wondered whether they also affect MMP-2, MMP-9 and uPA production

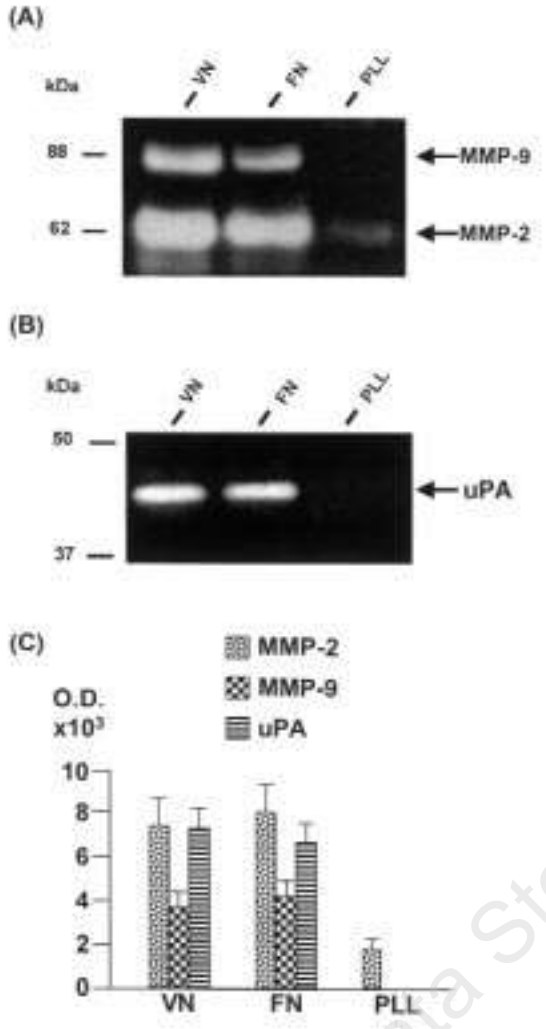

Figure 4. Increase of MMP-2, MMP-9 and uPA release by VN and FN in myeloma plasma cells. A) Fresh bone marrow plasma cells from one representative patient were incubated for 24 h at 37°C in SFM alone (control) or SFM to which VN, FN or PLL had been added. Conditioned media were analyzed by SDS-PAGE gelatin-zymography. White bands against a dark background correspond to the gelatinolytic areas of MMP-2 and MMP-9 activity. MMP-2 and MMP-9 are present in their cleaved form of 62 and 88 kDa respectively. B) Plasma cells were incubated for 24 h at 37°C in SFM admixed with VN, FN or PLL. Conditioned media were analyzed by SDS-PAGE casein-agarose zymography. White bands against a dark background correspond to the caseinolytic areas of uPA activity. C) Band intensity was quantified by computerized image analysis of the gel and expressed in arbitrary optical density (O.D.) units (mean ± 1 SD) in the 14 patients.

and release in myeloma plasma cells. These were incubated in suspension with VN and FN, and the activity of MMPs and uPA present in their CM was evaluated by gelatin- and casein-zymography, respectively.

Table 1. Effect of VN and FN on plasma cell proliferation.

Sample	*Number of plasma cells (× 10³)*		
	24 h	*48 h*	*72 h*
Positive control	20±3	40±6	85±14
Negative control	10±2	11±2	9±1
PLL	10±1	13±2	12±3
VN	14±5*	28±9†	58±10‡
FN	18±4*	32±8†	56±11‡
VN + FN	20±8*	42±16†	74±18‡
VN + anti-$\alpha_v\beta_3$	12±1	16±3	23±5
FN + anti-$\alpha_v\beta_3$	11±1	15±5	26±4
VN + FN + anti-$\alpha_v\beta_3$	14±4	21±6	25±6

** p<0.05, † p<0.01, and ‡ p<0.001 by Wilcoxon's rank test, as compared to the negative control or PLL-treated samples. Data are expressed as mean ± 1 SD of three determinations per patient in the 14 patients.*

As shown in Figure 4A, two gelatinolytic bands with Mr equal to 62 kDa and 88 kDa were detected. These bands correspond to the activated (or cleaved) forms of MMP-2 and MMP-9, respectively.[28,34] The bands were much fainter for MMP-2 (constitutive secretion) or absent for MMP-9 in the cells incubated with SFM alone or admixed with PLL (control). Computerized image analysis of the bands obtained in 6 independent experiments demonstrated that the increase of released MMP-2 and MMP-9 activity in VN- or FN-treated cells was reproducible and statistically significant for all the patients studied ($p<0.01$, Wilcoxon's rank test; Figure 4C). Moreover, MMP-2 and MMP-9 upregulation by VN and FN was totally inhibited by co-incubation with the neutralizing anti-$\alpha_v\beta_3$ antibody (*data not shown*).

When the CM were tested for uPA activity, those of PLL-treated plasma cells displayed no caseinolytic band, whereas one was present (45 kDa) in the CM of cells incubated with VN and FN (Figure 4B). No band was observed in the absence of plasminogen in the casein gel (*data not shown*), thus confirming its identity with uPA.

Discussion

Here we illustrate the ability of fresh bone marrow plasma cells from MM patients to interact with immobilized VN and FN. This interaction allows their prompt adhesion to the substratum. Total inhibition of this adhesion by a neutralizing monoclonal anti-$\alpha_v\beta_3$ integrin antibody indicates that $\alpha_v\beta_3$ integrin is the major receptor involved in this interaction. Cell interaction with ECM components, including VN and FN, gives rise to cytoskeleton organization and formation of plaques via integrin

clustering, followed by recruitment of cytoskeletal components and signaling molecules on the cytoplasmic side of the adhesion site. This interaction induces swift phosphorylation of the tyrosine kinase FAK with the formation of an SH_2 site for binding proteins of the Src family. Activation of $pp60^{src}$ leads to the phosphorylation of additional tyrosines in FAK that will bind the molecular adapter Grb-2/Sos complex and trigger the Ras/MAP kinase signal transduction cascade.[35,36] This results in activation of nuclear transcription factors, such as ETS-1,[37] which are the final mediators of the integrin transduction pathway. In human melanoma and breast carcinoma cells, this pathway governs important activities for tumor progression and spreading, including cell proliferation, migration, and production of ECM-degrading enzymes.[15-20] Accordingly, $\alpha_v\beta_3$ expression appears to be related to the progression of melanoma, breast, colon and ovary carcinomas and neuroblastic tumors.[19,20,38-40]

Our data show that fresh bone marrow plasma cells from MM patients interact with VN and FN, and that this interaction enhances cell proliferation and protease production and release. Interaction with VN or FN rapidly induces the recruitment of the β_3 integrin subunit, the cytoskeletal components vinculin and paxillin, the tyrosine kinases FAK and $pp60^{src}$, the small adapter protein Grb-2, and the MAP kinase ERK-2 in its phosphorylated form. Interaction with VN or FN also results in an increase in MMP-2, MMP-9 and uPA secretion. The substantial inhibition of all these effects by a neutralizing anti-$\alpha_v\beta_3$ integrin antibody points to integrin engagement in their regulation. Our previous study[22] showed that similar signal transduction pathways are operative in human lymphoid tumor cells adhering to VN and FN. Also, Podar *et al.*[41] have shown that vascular endothelial growth factor stimulates migration of an MM cell line and cell attachment to FN via β_1 integrin and protein kinase Cα activation. Overall data imply that cell attachment to the ECM proteins and subsequent stimulation of cell proliferation, migration and secretion of proteolytic enzymes is governed by several integrin complexes in MM.

We have observed that plasma cells proliferate when incubated in suspension with free VN or FN. This effect is additive. VN *plus* FN stimulation results in an increase in cell proliferation similar to that observed in serum-enriched medium. Interestingly, cells also proliferate under serum-free conditions when allowed to adhere to VN- or FN-coated plastics, indicating that both free and immobilized ECM proteins can modulate their growth.

Our data suggest that $\alpha_v\beta_3$ integrin engagement may also be of importance in the spreading of human MM. The increased protease export and proliferative capacity induced by the VN and FN interaction probably augment the ability of plasma cells to invade through the ECM of the perivascular stroma and subendothelial basement membrane. This, along with their increased angiogenic capacity[21] and induction of the vascular phase in the active disease,[42] may explain the frequent intramedullary and extramedullary dissemination of active MM.

Our data also suggest that inhibition of the $\alpha_v\beta_3$ integrin signal transduction pathway may have therapeutic potential in the treatment of patients with MM. Accordingly, tyrosine-kinase inhibitors, such as EGFR inhibitors, bradykinin antagonists, bcr/abl inhibitors and erb receptor inhibitors are envisaged for therapy of both hematologic tumors, such as chronic myeloid leukemia,[43] and Hodgkin's lymphoma,[44] and solid tumors, such as lung,[45] colorectal,[46] and breast carcinoma.[47] Lastly, the selective block of $\alpha_v\beta_3$ integrin with a humanized monoclonal antibody (Vitaxin)[48] or antisense oligonucleotides[49] as well as the inhibition of MMPs[50] may represent new therapeutic strategies for the treatment of MM.

Contributions and Acknowledgments

RR was primarily responsible for this paper, from conception to submitted manuscript. The other authors, listed according to a decreasing individual contribution to the paper, were responsible for the laboratory experiments, interpretation of data and statistical analyses. The last author had a major role as senior author in interpreting the data and preparing the article.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Thomas J. Kelly, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Prof. Kelly and the Editors. Manuscript received April 15, 2002; accepted June 11, 2002.

What is already known on this topic

Multiple myeloma is a plasma cell malignancy that is characterized by multiple lesions and is often accompanied by lytic bone disease. One question in multiple myeloma is how do the plasma cells disseminate from the site where the original transformed cell arose to multiple lesion sites throughout the body? One possibility is that the malignant cells disseminate in a manner similar to that known for solid tumors. That is, they use proteases to breakdown matrix barriers, adhesion molecules such as integrins to adhere to the matrix, and cytoskeletal machinery to migrate and invade into new areas.

What this study adds

This study supports the hypothesis that myeloma cells can disseminate by using $\alpha_v\beta_3$ integrins to adhere to the extracellular matrix molecules vitronectin and fibronectin. The work shows that once myeloma cells are attached to these matrix molecules, signals are transmitted through the grb-2, ERK-2 pathway, and increased proliferation as well as release of the proteases uPA, MMP-2 and MMP-9 is observed.

Potential implications for clinical practice

$\alpha_v\beta_3$ integrin, the proteases, or the signal transduction molecules, may be an appealing target for therapies to treat multiple myeloma.

Thomas J. Kelly, Associate Editor